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SYMPOSIUM: MICROBIOLOGY OF CHEESE FLAVOR DEVELOPMENT

Genetics of Proteolytic Enzymes of Lactococci and Their Role in Cheese Flavor Development

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ABSTRACT

In recent years, knowledge of the genetics and biochemistry of the enzymes that constitute the proteolytic system of starter lactococci has increased tremendously. This paper summarizes the data obtained largely in the last 5 yr of intensive research by various research groups operative in this field. The emphasis is on proteinase and peptidase enzymes and their genes and on the manipulation of proteolytic capacity of the bacteria by genetic techniques. The prospect of such genetically engineered strains in cheese fermentation and the first results in this area are discussed. (**Key words**: lactococci, proteolytic enzymes, cheese flavor, genetics)

Abbreviation key: MW = molecular weight.

INTRODUCTION

The proteolytic system of lactococci is crucially important for the growth of these fastidious organisms in milk. Proteinases and peptidases act in concert to degrade casein into amino acids and small peptides that are needed by the cells. In addition, the action of several proteinases and peptidases of lactococci is thought to play a role in the flavor development of fermented milk products. The actual contribution of various enzymes in flavor development has been difficult to assess because of the complexity of the system. In the last few years, an increasing number of proteinases and peptidases has been purified to homogeneity, thus allowing a detailed analysis of the specificity of the enzymes. Moreover, the availability of antibodies against, and N-terminal amino acid sequences of, the enzymes enabled the cloning of the structural genes of a number of peptidases. These recent developments in both the genetic and biochemical fields will ultimately lead to a better understanding of the intricate processes that underlie casein degradation and the liberation of both desirable and undesirable flavor peptides.

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THE CELL ENVELOPE-ASSOCIATED PROTEINASE

Most lactococcal strains used in dairy fermentations need an external supply of at least 10 amino acids to grow in milk. Because the concentration of free amino acids in natural milk is low and because casein is the major amino acid source, these bacteria depend on a proteinase to degrade casein into smaller peptide fragments, which can either be taken up directly or are substrates for further attack by various peptidases. Compared with some other bacterial species, lactococci are not very active proteolytically, but they carry an intricate array of a cell envelope-associated proteinase and various peptidases that enable them to grow rapidly in milk. Thus, lactococci lower the pH in milk, preventing the outgrowth of spoilage bacteria, and perform metabolic activities that ultimately determine the flavor of the cheese.

Lactococci Contain One Cell Envelope-Associated Proteinase (Caseinase)

Lactococcal proteinases have been the subject of intensive research because they may play an important role in cheese flavor development. Early studies [reviewed in (31, 43, 44)] indicated that the enzymes are located in the cell wall of most strains. Only one wild-type strain, *Lactococcus lactis* ssp. *cremoris* ML1, has been described that secretes a proteinase (31, 43, 44). Biochemical and immunological analyses, performed with either whole

cells or (partly purified) proteinase preparations, led to the classification of the proteinase by activity, specificity, and immunological properties (9, 14, 30, 51). The general understanding that emerged from these studies indicated the existence of a rather complex collection of different proteinases in the various strains examined, even within one strain of *Lactococcus*. Cloning and nucleotide sequence analysis of the proteinase genes revealed that the proteinase complex was less multifarious than expected (8, 20, 23, 24); this topic has been extensively reviewed by Kok (21). The model developed by Kok et al. (22), based on these genetic data, which explains the observed complexity in terms of enzyme instability and the occurrence of proteolytically active breakdown products of a large mature proteinase, has proven over time to be correct (21, 28, 29, 38). Lactococci clearly produce only one extracellular proteinase that can differ among the various strains with respect to specificity of casein breakdown. Proteinases truncated at the C-terminus have been constructed by genetic techniques and were proteolytically active: large parts of the C-terminus of the proteinase could be deleted without loss of proteolytic activity or specificity (22). The immunologically different proteins (proteinases) in the proteolytic system of lactococci are, in fact, conformationally different forms of the same proteinase recognized by the antibody preparation used.

The observation that proteinases truncated at the C-terminus are secreted in the growth medium, but that the wild-type proteinase is attached to the cells, led to the discovery of a membrane anchor in the extreme C-terminus of the proteinase. A hydrophobic stretch of 18 amino acids, flanked by an N-terminal Pro residue and 3 basic amino acids, shows similarities to membrane anchor sequences found in A, G, and M6 proteins of certain streptococci (11, 20, 53).

Similarities of Lactococcal Proteinase and Subtilisins

The nucleotide sequence of the proteinase gene of *L. lactis* ssp. *cremoris* Wg2 revealed that the enzyme is a member of the subtilisin family of serine proteinases (23). The similarity of the lactococcal proteinase with *Bacillus* subtilisins is most pronounced around those residues that form the active site and substrate-binding regions of the subtilisins. These findings agreed well with the observed sensitivity of the lactococcal enzymes to serine proteinase inhibitors. The most marked difference between the lactococcal proteinase gene (*prtP*) and the subtilisin genes is the size: the proteinase gene of strain Wg2 contains 1902 codons, whereas the subtilisin genes only contain 381 or 382 codons. The N-terminus of the primary translation product of *prtP* contains a typical signal peptide sequence of 33 amino acids. The subtilisins are produced as pre pro enzymes; the lactococcal enzymes are also similar in this respect. The N-terminal amino acid sequence of three mature lactococcal enzymes has been determined and, in all cases, was shown to be Asp 188 of the primary translation product (20, 28, 53). The pro region thus contains 155 amino acids, which is, again, much larger than that of the subtilisins.

Lactococcal Proteinase Maturation: an Autoproteolytic Process

Immediately upstream of *prtP*, a gene is present in the opposite orientation. This gene, *prtM*, is essential for proteinase activity (11, 53). PrtM is a 33-kDa lipoprotein that is located on the outside of the lactococcal cell membrane (12, 40). It shares similarity with *Bacillis subtilis* PrsA, a protein involved in secretion of exoproteins in this organism (26, 27). When *prtM* is deleted, and the cells carry intact *prtP*, they are unable to grow in milk. The cells produce proteinase, but the protein is larger than the proteinase produced in the presence of PrtM.

Apparently, PrtM is involved in the maturation of the proproteinase into the smaller mature active form. Haandrikman et al. (13) made an active site mutant of the lactococcal proteinase and showed that PrtM itself does not perform the proteolytic step that results in the formation of mature proteinase. In the current model of lactococcal proteinase maturation, PrtM is postulated to induce a conformational change in the proproteinase such that it is able to cleave its own proregion (21).

Genetic Manipulation of Proteinase Specificity

The proteinases of lactococci can be divided into two classes according to their specificity

of casein degradation (51). The so called PI (HP-type) proteinases degrade only β-casein, whereas the PIII (AM1-type) proteinases attack α_{s1}-, β-, and κ-casein. The β-casein degradation products of the PI and PIII enzymes are distinctly different. The availability of the genes of a PI and a PIII proteinase prompted a search for amino acids that were different among the proteinases that would be responsible for the difference in specificity (52). Two regions contained amino acids that contributed to the specificity of the proteinase: the region with homology to the substrate-binding site in subtilisin and, surprisingly, a stretch of amino acids outside of the "subtilisin core" of the lactococcal proteinase. That region contains two amino acid differences between the proteinase of the lactococcal strains Wg2 and SK11: Arg 747 and Lys 748 in the SK11 enzyme (PIII-type) are replaced by Thr and Gln residues in the proteinase of Wg2 (PI-type). Some of the hybrid proteinase constructed during this work had new casein breakdown specificities that were different from those of either of the wild-type proteinases.

Bacillus subtilis Neutral Proteinase Gene Expression in *Lactococcus lactis*

The isolation and characterization of lactococcal gene expression signals and the development of gene expression vectors for lactococci (48, 50) add a new dimension to the manipulation of proteolysis by lactococci. With these tools, the neutral proteinase from *B. subtilis* could be expressed in *L. lactis* (47). The recombinant strain secretes the bacillar proteinase, which seems to be correctly processed at its N-terminus to give the mature active proteinase, and is proteolytically more active than a strain carrying the lactococcal *prt* genes.

Insertion of *prt* Genes in the Chromosome of *L. lactis*

A methodology for the integration of genetic information in the chromosome of lactococci has been developed recently (33). The strategy is based on a plasmid that does not replicate in *L. lactis* and is endowed with a gene that can be directly selected in *Lactococcus*. When a piece of chromosomal DNA is inserted into this vector and the resulting plasmid is introduced in *Lactococcus*, it will integrate in the chromosome at the site of homology between chromosome and plasmid. This technique has been used to secure the proteinase genes in the chromosome of *Lactococcus* (32). Two strains were obtained: with 2 and with 8 copies of the *prt* genes in the chromosome. The proteolytic activity of the strains corresponded to the copy number of the *prt* genes. Both strains grew rapidly in milk with rapid acid production, and the stability of the proteinase-positive phenotype in milk was far superior to a strain carrying the *prt* genes on a plasmid. Whereas 50% of the plasmid-carrying cells lost the proteinase-positive phenotype within 20 generations, no loss of proteolytic ability was observed for at least 100 generations in cells carrying the *prt* genes on the chromosome.

PEPTIDASES

After the initial description of various peptidase activities in lactococci by examination of (partially purified) cell-free extracts in the late 1970s and early 1980s, emphasis has more recently shifted to the purification to homogeneity of individual peptidases. Presently, 10 peptidases have been purified from different strains of *Lactococcus*. Results are summarized in Table 1 and briefly described herein.

Petidase Purification

Two distinct broad-specificity aminopeptidases, PepN and PepC, have been isolated from *L. lactis* ssp. *cremoris* strains Wg2 and AM2, respectively (37, 41). Both enzymes hydrolyze a large number of amino acyl-*para*-nitroanilides and several dipeptides, tripeptides, and larger peptides by removal of the N-terminal amino acid. Aminopeptidase PepN is a metalloenzyme that is irreversibly inhibited by EDTA, whereas PepC is inhibited by sulfhydryl group inhibitors. Purified PepN has a molecular mass of 95 kDa; PepC is 50 kDa by SDS-PAGE and may form a hexamer of 300 kDa under native conditions.

Several Pro-specific peptidase activities have been characterized in lactococci. A 43-kDa prolidase (Pro dipeptidase) specifically

TABLE 1. Lactococcal proteolytic enzymes purified to homogeneity.[1]

Enzyme	Pure	Monomer[2]	Class	Substrate	nt seq	Leader peptide	Location
PrtP	Y	200	Serine	CN	Y	Y	Envelope
Neutral proteinase		93	Neutral	β-CN			in
PepA	Y	43	Metallo	Glu- and Asp-*p*NA			in and E
PepC	Y	50	Thiol	Leu- and Lys-*p*NA	Y	N	in and C
PepN	Y	95	Metallo	Leu- and Lys-*p*NA	Y	N	in and C
PepXP	Y	90	Serine	X-Pro-*p*NA	Y	N	in and M
PCP		. . .	Serine	PyroGlu-*p*NA			in
Dipeptidase	Y	49	Metallo	Leu-Leu			in and ex
Tripeptidase	Y	52	Metallo	Tripeptides			in and M
Prolidase	Y	43	Metallo	X-Pro			in
Iminopeptidase	Y	50	Metallo	Pro-X-(Y)			in
LEPI	Y	93	Metallo	α_{s1}-CN (f1-23)			Cell wall
PepO	Y	70	Neutral	α_{s1}-CN (f1-23)	Y	N	in and M

[1]Y = Yes; N = no; in = intracellular; ex = extracellular; nt seq = nucleotide sequence known; E = envelope; C = cytosol; M = membrane; CN = casein; *p*NA = *p*-nitroanilide.

[2]Molecular weight × 10^3.

degrading X-Pro peptides has been purified to homogeneity from *L. lactis* ssp. *cremoris* H61 (17). The enzyme is completely inhibited by the metal chelators 1,10-phenantroline and EDTA. A Pro iminopeptidase purified from the cytoplasm of *L. lactis* ssp. *cremoris* HP has a monomeric molecular mass of approximately 50 kDa (3). The enzyme degrades dipeptides and tripeptides with Pro as the N-terminal amino acid, but not the prolidase substrates X-Pro. The enzyme seems to be common in lactococci and was found in all strains tested (3). An enzyme liberating X-Pro dipeptides from larger peptide fragments, X-prolyl dipeptidyl aminopeptidase (PepXP), is generally present in lactococci and in all species of lactic acid bacteria (1, 4, 7, 18, 19, 35, 55). All lactococcal PepXP are severely inhibited by the serine proteinase inhibitors phenylmethylsulfonylfluoride and diisopropylfluorophosphate. The monomers range in size from 80 to 95 kDa (as estimated by SDS-PAGE), whereas the enzymes form dimers of 160 to 200 kDa under native conditions. The PepXP enzyme from *L. lactis* ssp. *cremoris* AM2 is unusual and was reported to have a size of 117 kDa by Sephadex G-200 gel filtration (4).

Two broad-specificity dipeptidases and a tripeptidase have been isolated and extensively analyzed. A true dipeptidase from *L. lactis* ssp. *cremoris* H61 has a native molecular weight (MW) of 100,000 and hydrolyzes various dipeptides if the N-terminus of the peptides is not blocked, but does not attack tripeptides (15, 16). The dipeptidase of *L. lactis* ssp. *cremoris* Wg2 has a MW of 46,000 under native and denaturing conditions (46). Both enzymes are metallopeptidases because they are both severely inhibited by EDTA and 1,10-phenantroline. The tripeptidase of strain Wg2 was purified to homogeneity and shown to exist as a dimer of two identical 52-kDa subunits under native conditions (5). The enzyme hydrolyzes a large selection of tripeptides by splitting off the N-terminal amino acid residue, but does not attack dipeptides and oligopeptides.

In addition to the exopeptidases already mentioned, several endopeptidases have recently been purified from lactococci. Endopeptidase LEP-I from *L. lactis* ssp. *cremoris* H61 is active as a 98-kDa monomer and was purified using its activity on α_{s1}-casein (f1-23) (54). This α-casein fragment and α_{s1}-casein (f91-100) are hydrolyzed at their Glu-Asn bonds as long as they are not part of larger peptides. Some small peptide hormones are also degraded and are cleaved at peptide bonds other than the Glu-Asn bond. An endopeptidase from strain Wg2 was purified to homogeneity using metenkephalin as a substrate (42). The enzyme splits the Gly-Phe bond in this peptide and has a broad substrate specificity. It degrades several peptide hor-

mones and β-casein fragments, but not α-, β-, or κ-casein or peptides smaller than 5 amino acids. The endopeptidase is 70 kDa by both SDS-PAGE and gel filtration.

Peptidase Localization

One important, recurring problem in the analysis of the proteolytic system of lactococci is the cellular location of the enzymes. Biochemical and genetic data clearly indicate that the lactococcal proteinase is bound to the cell by its C-terminal membrane anchor, from which it probably extends over the cell wall into the surrounding medium where it finds its substrate, casein. Present understanding of the location of the peptidolytic enzymes is incomplete because proper controls for cell lysis during cell fractionation studies have not been rigorously applied. The isolation of specific antibodies would help greatly to resolve this problem; that has been achieved in the past 2 yr through the purification to homogeneity of various peptidases. Tan et al. (40) used polyclonal antibodies raised against PepN, PepC, PepXP, the tripeptidase of strain Wg2, and the 70-kDa endopeptidase in cell fractionation studies combined with proteinase K accessibility experiments and in immunogold labeling studies. Those authors concluded that all five peptidases were located intracellularly because they were not sensitive to proteinase K digestion. Immunogold studies indicated that PepXP, tripeptidase, and endopeptidase are located at the cell periphery, which is in accordance with their cofractionation with cell membranes. The location of another peptidase, PepA, is still somewhat obscure although an intracellular, membrane-bound location seems to fit the present data best (2, 10).

Nucleotides, Codons, and Amino Acids

The genes of four of the peptidases have so far been isolated and sequenced to completion. The *pepXP* genes of *L. lactis* ssp. *cremoris* P8-2-47 and *Lactococcus lactis* ssp. *lactis* 763 have been cloned by using peptidase activity complementation strategies (34, 36). The gene of strain P8-2-47 was cloned in *Escherichia coli* by making use of the fact that this organism does not normally contain PepXP activity. A chromosomal DNA fragment carrying the lactococcal *pepXP* gene, which was inserted into an *E. coli* vector and introduced into *E. coli*, endowed the strain with this new activity, which could be easily visualized by a chromogenic plate assay. The *pepXP* gene of strain 763 was cloned by complementation of an *L. lactis* ssp. *lactis pepXP* mutant. Both PepXP are virtually identical and are not similar to any other known protein. A comparison of the deduced amino acid sequences of the PepXP with the N-termini of the purified peptidases shows that the enzymes are not N-terminally processed.

Aminopeptidase PepC of *L. lactis* ssp. *cremoris* AM2 was cloned by complementation of an *E. coli pepN* mutant using an enzymatic plate assay (56). The *pepC* gene contains 436 codons, and only the initiation Met is cut off from PepC; PepC shares similarities with Cys proteinases, such as papain, especially in the active site of the latter.

The other two peptidases, PepN and the 70-kDa endopeptidase, were cloned by use of antibodies to screen λ-phage chromosomal DNA libraries. The *pepN* gene contains 846 codons, and the deduced MW of PepN of 95,300 is in good agreement with that determined biochemically (39, 45). Peptidase PepN is homologous to eukaryotic and bacterial Zn-metallo aminopeptidase N. One of the homologous regions is similar to a region in thermolysin that is part of the active site and contains an essential ion-binding site for Zn.

With antibodies raised against the purified 70-kDa endopeptidase, a λ-phage expressing the enzyme from *L. lactis* ssp. *cremoris* P8-2-47 was isolated and used to subclone the endopeptidase gene, *pepO*, in an *E. coli* plasmid (I. Mierau, 1992, personal communication). The gene was sequenced completely and contains 627 codons. The deduced MW of PepO (71,500) agrees with that determined by SDS-PAGE. No promoter was apparent immediately upstream of *pepO*; instead, another open reading frame was present on the same DNA strand. Subcloning experiments in *E. coli* showed that a promoter upstream of this open reading frame is actually used to transcribe *pepO*. Again, the N-terminus of the primary translation product of *pepO* is the same as that of the purified enzyme, showing that PepO is not N-terminally processed. The enzyme has similarities to mammalian neutral endopepti-

dases, especially in the active site and Zn-binding domain of the enkephalinases.

Peptidase Mutants and Their Performance in Cheese Making

Both chemical mutagenesis and the plasmid integration strategy described were used to make mutations in a number of the peptidase genes presently isolated. With these mutants, the importance of the peptidases for growth in milk was assessed. Peptidases PepN, PepXP, and PepO were not essential for growth in milk (B. Mayo, I. Mierau, A. J. Haandrikman, and K. L. Leenhouts, 1992, personal communication). Two chemically induced mutants, a *pepN* strain and a strain mutated in *pepXP*, were used in cheese-making trials to examine the role of the peptidases in cheese flavor development (2). Gouda-type cheeses were made with .25% starter cultures of varying composition. All starters contained 10% proteolytically active cells of *L. lactis* ssp. *cremoris* HP, which is a "bitter"-producing strain, and 90% proteinase-negative cells. The latter fraction consisted of cells from strain HP and increasing concentrations of either the *pepN* or the *pepXP* mutant. Organoleptic quality, amino acid N, and salt-soluble N were examined. The extent of peptide hydrolysis was estimated by reversed-phase HPLC. Higher concentrations of *pepN* mutants in the starter culture led to increased bitterness, which was noticeable after 2 wk of ripening and was ascribed to the accumulation of low MW peptides that are normally degraded by PepN of wild-type starter strains. Cheeses manufactured with increasing concentrations of the PepXP-deficient strain did not accumulate bitterness but exhibited decreasing organoleptic quality.

CONCLUSIONS

The development of techniques to manipulate lactococci genetically in the mid-1980s has been crucial to current understanding of proteolysis by lactococci. Genetic, biochemical, and immunological strategies provided extensive information on the make-up of the proteolytic system of lactococci. The proteinase structural gene, *prtP*, encodes a very large pre pro proteinase. A second protein, PrtM, the gene of which is always tightly coupled to *prtP*, induces the proproteinase to perform an autoproteolytic activation step. Both PrtP and PrtM are attached to the cell membrane: PrtP with its C-terminal membrane anchor and PrtM through an N-terminal, lipo-modified Cys residue.

Knowledge of the nucleotide sequence of *prtP* allowed the in vitro manipulation of proteolytic activity of lactococcal cells in three ways. First, the genes for PrtP and PrtM could be permanently inserted in the chromosome of *L. lactis*, thereby solving the inherent instability of proteolysis that is due to the plasmid nature of the *prt* genes. Second, exchange of DNA fragments representing different regions of the proteinase molecule has indicated which amino acids are important in determining proteinase specificity. Hybrid proteinases have been constructed exhibiting novel caseinolytic specificities. Third, the proteolytic activity of *Lactococcus* sp. could be enhanced by increasing the copy number of the *prt* genes, either by moving the genes from their original location on plasmids with a low copy number to vectors with a moderate copy number (6, 25) or by chromosomal insertion and subsequent gene amplification (32). In a different approach, the *prtP* and *prtM* genes were rearranged in an operonlike structure and endowed with a promoter that was stronger than the original *prt* promoters (49).

The number of different enzymes in the peptidolytic system that have been purified to homogeneity has increased rapidly. The purification allowed the production of (polyclonal) antibodies against each of these peptidases and the determination of the N-terminal amino acid sequences of a number of them. Both the N-terminal amino acid sequence information and the antibodies can be used to clone out the respective peptidase genes and to examine them at the nucleotide level. This procedure will likely soon lead to extensive new information on peptidase gene regulation, on importance of the enzymes in cell physiology and cheese flavor development by the targeted construction of peptidase mutants, and on peptidase localization. This information will also allow the overproduction of peptidases in *L. lactis*, or in *E. coli*, for the isolation of the enzymes for detailed biochemical studies or for use in new (nondairy) applications.

Apart from the extracellular proteinase, extracellularly located peptidases are also thought to play an important role in cheese flavor development. Based on this assumption, several research groups set out to identify and to characterize such peptidases. Until the present, none of the peptidases analyzed meets the rigorous criteria that would allow the label "extracellular". All peptidases thus far examined—namely, the two general aminopeptidases PepN and PepC, PepXP, PepO, the 49-kDa tripeptidase of strain Wg2, and, although this case is less clear, PepA—are truly intracellular. The exact location within the cell is still somewhat obscure. The latter four peptidases may be located in or near the inner phase of the cell membrane, perhaps even attached to the membrane through other proteins. This observation and data showing that PepN and PepXP affect cheese quality show that intracellular peptidases may be highly important determinants in cheese ripening. Amino acids are now considered to be the prime flavor precursors in cheese. Amino acids undergo chemical or enzymatic conversion to essential flavor components, and the rapid accumulation of a pool of free amino acids early in the cheese-making process seems to be preeminent for full flavor development. This pool may be produced by (limited) lysis of cells, thus liberating the intracellular enzymes, or may even represent the intracellular amino acid pool of the starter cells (2). The lactococcal strain that secretes *B. subtilis* neutral proteinase might be useful in the rapid extracellular degradation of peptides to free amino acids; the introduction of that strain in cheese, perhaps together with multiple copies of pepN and pepXP to remove possibly accumulating bitter peptides, may lead to acceleration of cheese flavor development.

The real future challenge is to apply the accumulated knowledge on the proteolytic system in dairy practice. Such application experiments are currently underway, and the outcome, although still somewhat premature, is very promising. Both PepN and PepXP are important determinants in cheese flavor development, and manipulation of the activities of these peptidases may prove useful in the future. The effect on cheese quality of use of strains with genetically altered proteinases in cheese-making trials will be of particular interest. In all cases, however, development of strategies to manipulate lactococci genetically according to food-grade standards is imperative if genetically altered strains are to be used in foods. This development should receive more future attention because the tools are now available to construct improved *Lactococcus* strains.

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